PROCESS FOR PREPARING POLYHYDROXYALKANOATE EMPLOYING *maoC* GENE

The present invention relates to a method for producing polyhydroxyalkanoate (hereinafter, referred to as "PHA") using a *maoC* gene. More particularly, the present invention relates to a *maoC* gene, a MaoC protein having enoyl-CoA hydratase activity, and a method for producing PHA using a microorganism transformed with a recombinant vector containing the *maoC* gene and a recombinant vector containing a PHA synthase gene.

Background Art

PHA is a polyester-type compound, which is accumulated within a microorganism to act as a carbon source when the microorganism has excess carbon source present therein while it lacks other nutrients, such as phosphorus, nitrogen, magnesium and oxygen. Since this PHA has physical properties similar to synthetic polymer originated from petroleum while showing complete biodegradability, it is being recognized as a substitute for prior synthetic plastics.

Generally, PHA is divided into short-chain-length PHA (SCL-PHA) with a small number of carbon atoms and medium-chain-length PHA (MCL-PHA) with a relatively large number of carbon atoms. Since MCL-PHA has more similar physical properties to synthetic polymer than SCL-PHA, studies

on generally MCL-PHA are being actively conducted. To produce PHA in a microorganism, different enzymes are required: enzymes capable of converting metabolites of the microorganism into PHA monomers, and a PHA synthase capable of synthesizing a PHA polymer from the PHA monomers. The enzymes known to provide the PHA monomers include (R)-specific enoyl-CoA hydratase found in *Aeromonas* sp. microorganisms and *Pseudomonas* sp. microorganisms, and 3-ketoacyl-ACP reductase found in *E. coli* and *Pseudomonas* sp. microorganisms (Fukui et al., *J. Bacteriol.*, 180:667-73, 1998; Tsuge et al., *FEMS Microbiol. Lett.*, 184: 193-8, 2000; Taguchi et al., *FEMS Microbiol. Lett.*, 176: 183-90, 1999; Ren et al., *J. Bacteriol.*, 182:2978-81, 2000; and Park et al., *FEMS Microbiol. Lett.*, 214:217-22, 2002).

Meanwhile, it was reported that a MCL-PHA synthase gene was cloned from a *Pseudomonas* sp. microorganism, and MCL-PHA could be synthesized using a recombinant microorganism transformed with this gene (Qi et al., *FEMS Microbiol. Lett.*, 157:155-62, 1997; Qi et al., *FEMS Microbiol. Lett.*, 167:89-94, 1998; Langenbach et al., *FEMS Microbiol. Lett.*, 150:303-9, 1997; WO 01/55436; USP 6,143,952; WO 98/54329; and WO 99/61624). Furthermore, it was reported that the use of a recombinant *E. coli*, from which FadB among enzymes of fatty acid degradation pathway had been deleted, could also produce MCL-PHA (Langenbach et al., *FEMS Microbiol. Lett.*, 150:303-9, 1997).

On the other hand, since it was reported that YfcX enzyme, homologous to FadB could provide the PHA monomers when the *fadB* gene-deleted recombinant *E. coli* was used (Snell et al., *J. Bacteriol.*, 184: 5696-5705, 2002) it was predicted that new enzymes of providing the PHA monomers besides YfcX would exist. Because the use of the *fadB* gene-

deleted recombinant *E. coli* can effectively produce MCL-PHA, efforts to discover the enzymes of providing the PHA monomers in the *fadB* gene-deleted recombinant *E. coli* were continued but there were no particular results up to now.

Accordingly, there was a continued need to discover the new enzymes of providing the PHA monomers in *E. coli.*

Disclosure of Invention

Therefore, the present inventors have conducted extensive studies to discover a new enzyme of providing PHA monomers in *E. coli,* and consequently, found that a protein expressed from an *E. coli* gene *maoC* whose other functions had not been yet established, could serve as the enzyme of providing the PHA monomers in *fadB* gene-deleted recombinant *E. coli.* On the basis of this point, the present invention was perfected.

An object of the present invention is to provide a *maoC* gene, a MaoC protein, a recombinant vector containing the *maoC* gene, and a microorganism transformed with the recombinant vector.

Another object of the present invention is to provide a method for producing medium-chain-length PHA using the *maoC* gene.

To achieve the above objects, in one aspect, the present invention provides a *maoC* gene coding for a MaoC protein represented by SEQ ID NO: 1.

In another aspect, the present invention provides a *maoC* gene, which has a DNA sequence of SEQ ID NO: 2 and codes for a protein of providing monomers necessary for MCL-PHA synthesis.

In yet another aspect, the present invention provides a recombinant vector containing the *maoC* gene.

In still another aspect, the present invention provides a MaoC protein, which has an amino acid sequence of SEQ ID NO: 1 and shows enoyl-CoA hydratase activity.

In further another aspect, the present invention provides a microorganism transformed with the recombinant vector.

In the present invention, the microorganism is preferably deleted of a *fadB* gene and contains a PHA synthase gene. Also, the microorganism is preferably transformed with a recombinant vector containing the PHA synthase gene, or has the PHA synthase gene inserted into a chromosome.

In the present invention, the PHA synthase gene is preferably *phaC*.

In another aspect, the present invention provides a method for producing middle-chain-length PHA (MCL-PHA), which comprises the steps of: culturing the transformed microorganism in a medium containing a C_{6-10} carbon source; and obtaining MCL-PHA consisting of monomers with 6-10 carbon atoms.

Furthermore, the present invention provides MCL-PHA, which is produced as described above so that the content of each of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) in the total monomers of MCL-PHA is more than 30%.

Tsuge *et al.* reported enoyl-CoA hydratase involved in the synthesis of PHA in *Pseudomonas* sp. (Tsuge et al., *FEMS Microbiol. Lett.,* 184:193-8, 2000). To discover a new enzyme of providing PHA monomers in *E. coli,* the present inventors searched for proteins, which are expressed in *E. coli* and have an amino acid sequence showing high homology with that of the reported enoyl-CoA hydratase. As a result, a MaoC protein of SEQ ID NO: 1 showing

a homology of 34%, and a gene of SEQ ID NO: 2 coding for this protein, were discovered. Up till now the exact function of the MaoC protein was not established, and only the fact that each of *maoC* and *maoA* genes consists of one operon was reported (Steinebach et al., *Eur. J. Biochem.,* 237:584-91, 1996).

The present inventors predicted that the discovered protein would show enoyl-CoA hydratase activity involved in PHA synthesis. To confirm this, the *maoC* gene coding for the MaoC protein was cloned. In other words, using the *E. coli* chromosome as a template, a $maoC_{Ec}$ gene was amplified by PCR using an oligonucleotide primer synthesized on the basis of the genomic sequence of *E. coli* (Blattner et al., *Science* 277:1453-62, 1997). The amplified $maoC_{Ec}$ gene was digested with *Sac*I/*Xba*I and cloned into a p10499A recombinant vector, thereby constructing a p10499MaoC vector. A gene fragment obtained by digesting the p10499MaoC vector with *Eco*RV/*Sca*I was inserted into pACYC184 digested with *Pvu*II/*Dra*I, thereby constructing a pACYC104MaoC recombinant vector for expressing the *maoC* gene.

A p10499613C2 vector capable of expressing the PHA synthase gene was digested with *Eco*RV/*Ssp*I, and inserted into pBBR1MCS digested with *Eco*RV, thereby constructing a pMCS104613C2 recombinant vector containing the PHA synthase gene.

According to the method of Jeong and Lee, a *maoC* gene was deleted from mutant *E. coli* WB101 from which a *fadB* gene had been deleted, thereby producing mutant *E. coli* WB106 from which both the *fadB* and *maoC* genes had been deleted (Jeong and Lee., *Appl. Environ. Microbiol.,* 68:4979-85, 2002).

The mutant *E. coli* WB101 from which only the *fabB* gene had been deleted could produce MCL-PHA from decanoate with 10 carbon atoms even when it was transformed with pMCS104613C2 containing the PHA synthase gene, but the mutant *E. coli* WB106 from which both the *fadB* and *maoC* genes had been deleted could not produce MCL-PHA from decanoate with 10 carbon atoms when it was transformed with only pMCS104613C2. However, *E. coli* W3110 obtained by the transformation of *E. coli* WB106 with both pMCS104613C2 and pACYC104MaoC could produce MCL-PHA from decanoate with 10 carbon atoms.

Although medium for use in culturing of *E. coli* is not specially limited, it is preferred to use Luria-Bertani (LB) medium (yeast extract 5g/L, peptone 10g/L, NaCl 5g/L), which is generally used in culturing of *E. coli*. It could be found that PHA produced according to the present invention showed superior yield to PHA produced by the prior method, and contained 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as monomers, and particularly 3HO and 3HD formed most of the monomers. Thus, it was found that PHA produced by the present invention has superior quality to PHA produced by the prior method.

To measure the enzyme activity of the protein expressed from the *maoC* gene, recombinant vector pTac99MaoCH expressing the MaoC protein labeled with 6 histidines was constructed, and MaoC-His_6-Tag was obtained using the recombinant vector pTac99MaoCH. This MaoC-His_6-Tag showed an enoyl-CoA hydratase activity of 47.6 U/mg as measured in the presence of crotonyl-CoA as a substrate. As used herein, "1U" is defined as a unit of enzyme activity for removing 1 μmol of crotonyl-CoA per minute.

Brief Description of Drawings

FIG. 1 is a gene map of recombinant vector p10499MaoC containing a *maoC* gene;

FIG. 2 is a gene map of recombinant vector pACYC104MaoC containing a *maoC* gene;

FIG. 3 is a gene map of recombinant vector pMCS104613C2 containing a PHA synthase gene; and

FIG. 4 is a gene map of recombinant vector pTac99MaoCH containing a *maoC* gene.

Best Detailed Description of the Invention

The present invention will hereinafter be described in further detail by examples. It will however be obvious to a person skilled in the art that these examples are given for illustrative purpose only, and the scope of the present invention is not limited to or by these examples.

Example 1: Production of recombinant *E. coli* for producing PHA using *maoC* gene

Mutant *E. coli* from which *fadB* and *maoC* genes had been deleted, a recombinant vector containing an *E. coli maoC* gene, and a recombinant vector containing a MCL-PHA synthase gene, were constructed. Using each of these recombinant vectors, recombinant *E. coli* for use in the production of MCL-PHA was produced.

Example 1-1: Production of mutant *E. coli* from which *fadB* and *maoC* genes had been deleted

According to the conventional method, a *maoC* gene was deleted from *E. coli* using the *red* operon of bacteriophage λ (Jeong and Lee, *Appl. Environ. Microbiol.*, 68:4979-85, 2002).

In other words, mutant *E. coli* WB101 from which a *fadB* gene had been deleted was transformed with vector pTrcEBG (Jeong and Lee, *Appl. Environ. Microbiol.*, 68:4979-85, 2002) containing the *red* operon of bacteriophage λ, and added with 1mM IPTG to induce the expression of *red* operon in the *E. coli* WB101 transformed with pTrcEBG, and a electroporation-competent cell was produced using this *E. coli*. The *E. coli* WB101 was produced by deleting the *fadB* gene from *E. coli* W3110 (ATCC 39936) (Park et al., *Enzyme Micro. Technol.*, 33: 62-70, 2003).

Meanwhile, since 60 bp from the 5' end and 60 bp from the 3' end of the *maoC* gene contain DNA sequences similar to 60 bp from the 5' end and 60 bp from the 3' end of a chloromphenicol-resistant gene, the substitution of the *maoC* gene with the chloromphenicol-resistant gene can produce a *maoC* gene-deleted mutant. For this purpose, using pACYC184 (New England Biolab, USA) as a template and primers MaoCdelf (SEQ ID NO: 3) and MaoCdelb (SEQ ID NO: 4), 30 cycles of PCR consisting of denaturation at 94 °C for 50 seconds, annealing at 52 °C for 50 seconds and extension at 72 °C for one minute was carried out, thereby producing a PCR fragment.

5'-atgcagcagttagccagtttcttatccggtacctggcagtctggcc
ggggccgtagccgtagcacttcactgacaccctc-3' (SEQ ID NO: 3)

5'-ttaatcgacaaaatcaccgtgctgcctggccaccagcgtcagaatt gaataacttattcaggcgtagcacc-3' (SEQ ID NO: 4)

The PCR fragment was cloned into the electroporation-competent cell produced as described above, thereby constructing mutant *E. coli* WB106 from which both the *fadB* and *maoC* genes had been deleted.

Example 1-2: Construction of p10499A and p10499613C2

Using the chromosomal DNA of *Pseudomonas* sp. 61-3 (JCM 10015) containing a PHA synthase gene (Matsusaki et al., *J. Bacteriol.,* 180: 6459-67, 1998) as a template and primers phaC2$_{Ps}$-1 (SEQ ID NO: 5) and phaC2$_{Ps}$-2 (SEQ ID NO: 6), 30 cycles of PCR consisting of denaturation at 94 °C for 50 seconds, annealing at 52 °C for 50 seconds and extension at 72 °C for 2 minutes was carried out, thereby producing a PCR product.

5'-cgcggatccaataaggagatatctagatgagagagaaaccaacgccg-3' (SEQ ID NO: 5)
5'-cggatccccgggtaccgagctcgaattctcagcgcacgcgcacgtaggta-3' (SEQ ID NO: 6)

The analysis of the PCR product was conducted by agarose gel electrophoresis and showed a 1.7 kb gene fragment corresponding to a *phaC2Ps* gene.

Moreover, to amplify a *gntT104* promoter such that the PHA synthase gene is continued to express, 30 cycles of PCR consisting of denaturation at 94 °C for 50 seconds, annealing at 52 °C for 50 seconds and extension at 72 °C for 2 minutes was conducted using the chromosomal gene of *E. coli* W3110 (ATCC 39936) as a template and primers gntT104-1 (SEQ ID NO: 7) and

gntT104-2 (SEQ ID NO: 8) (Peekhaus and Conway, *J. Bacteriol.*, 180:1777-85, 1998).

5'-gactagttgaaaggtgtgcgcgatctcac-3' (SEQ ID NO: 7)

5'-gcggatcccatttgttatgggcgacgtcaattt-3' (SEQ ID NO: 8)

The analysis of the PCR product was conducted by agarose gel electrophoresis and showed a 400 bp gene fragment.

Plasmid pTrc99A (Pharmacia Biotech. Co., Uppsala, Sweden) was digested with EcoRV/EcoRI, and then, a *gntT104* promoter gene fragment digested with the same enzymes was ligated to the pTrc99A plasmid by T4 DNA ligase, thereby a p10499A vector (Park et al., *FEMS Microbiol. Lett.*, 214: 217-22, 2002). Then, the p10499A vector was digested with EcoRI/HindIII, after which the amplified $phaC2_{Ps}$ gene was inserted into EcoRI/HindIII recognition sites of the p10499A vector, thereby constructing recombinant expression vector p10499613C2.

Example 1-3: Construction of recombinant vector containing *maoC* gene

First, the chromosomal DNA of *E. coli* W3110 was isolated and purified according to a known method (Sambrook et al., *Molecular cloning*, 2nd ed, Cold Spring Harbor Laboratory Press, NY, 1989). Using the purified *E. coli* genomic sequence as a template, and primers *maoC* 1 (SEQ ID NO: 9) and *maoC* 2 (SEQ ID NO: 10), 30 cycles of PCR consisting of denaturation at 94 °C for 50 seconds, annealing at 52 °C for 50 seconds and extension at 72 °C for 2 minutes was conducted, thereby giving a PCR fragment.

5'-tttcccgagctcatgcagcagttagccagtt-3' (SEQ ID NO: 9)

5'-gctctagattaatcgacaaaatcaccgt-3' (SEQ ID NO: 10)

Meanwhile, the p10499A vector was digested with *SacI/Xba*I and then inserted with the PCR fragment, thereby constructing recombinant vector p10499MaoC. The p10499MaoC recombinant vector was digested with *Eco*RV/*Sca*I, cloned into pACYC184 digested with *Pvu*II/*Dra*I, thereby constructing recombinant vector pACYC104MaoC (see, FIGS. 1 and 2). FIG. 1 is a gene map of the recombinant vector p10499MaoC containing the *maoC* gene, and FIG. 2 is a gene map of the recombinant vector pACYC104MaoC containing the *maoC* gene.

Example 1-4: Construction of recombinant vector containing MCL-PHA synthase gene

For expression of a MCL-PHA synthase gene, a gene fragment obtained by digesting p10499613C2 with *Eco*RV/*Ssp*I was cloned into pBBR1MCS (Kovach et al., *Gene,* 166: 175-6, 1995) digested with *Eco*RV, thereby constructing MCL-PHA synthase gene recombinant vector pMCS104613C2 (see, FIG. 3). FIG. 3 is a gene map of the recombinant vector pMCS104613C2 containing the PHA synthase gene.

Example 1-5: Production of recombinant *E. coli*

The mutant *E. coli* WB101 from which the *fadB* gene had been deleted, and the mutant *E. coli* WB106 from which both the *fadB* and *maoC*

genes had been deleted, were transformed with the MCL-PHA synthase gene recombinant vector pMCS104613C2 and the MaoC recombinant vector p10499MaoC, and the MaoC recombinant vector pACYC104MaoC, thereby producing recombinant *E. coli* strains WB101 (transformed with pMCS104613C2), WB101 (transformed with pMCS104613C2 + p10499MaoC), WB106 (transformed with pMCS104613C2) and WB106 (transformed with pMCS104613C2 + pACYC104MaoC). As a control group, recombinant *E. coli* W3110 was used, which had been produced by transformation of normal *E. coli* W3110 with the PHA synthase gene recombinant vector pMCS104613C2 and the MaoC expression recombinant vector p10499MaoC.

Example 2: Measurement of MCL-PHA-synthesizing capability of recombinant *E. coli*

In order to measure the MCL-PHA-synthesizing capability of the control group *E. coli* strain, and the test group *E. coli* strains WB101 (pMCS104613C2), WB101 (pMCS104613C2 + p10499MaoC), WB106 (pMCS104613C2) and WB106 (pMCS104613C2 + pACYC104MaoC) produced in Example 1-5, each of the recombinant *E. coli* strains was cultured in LB medium (yeast extract 5g/L, peptone 10g/L, NaCl 5g/L) containing 2 g/l of decanoate for 4 days. Then, the culture broth was centrifuged at 2,500rpm for 15 minutes, and dried at 100 °C. According to the conventional method, MCL-PHA was isolated from the dried colony and measured for its content in the colony.

In order to measure the composition of the isolated MCL-PHA, the isolated MCL-PHA was subjected to methanolysis to convert it into the form

of 3-hydroxyalkanoic acid methyl ester, and the composition ratio between 3-hydroxybutyrate (3HB), 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD) was measured by gas chromatography (Donan Co., Korea). In this case, a fused silica capillary column (Supelco SPBTM-5, 30m 0.32mm ID 0.25m film, USA) was used as a GC column, and benzoic acid (Sigma Chem. Co., USA) was used as the internal standard. The measured results are given in Table 1 below.

Table 1. Content and composition ratio of MCL-PHA in recombinant *E. coli.*

Test group	MCL-PHA content (%, w/w)	Composition ratio (mol%)				
		3HB	3HHx	3HO	3HD	3HHD
Control	11.9	0	19	74	7	0
WB101 (pMCS104613C2)	44.6	0	9	37	54	0
WB101 (pMCS104613C2 + p10499MaoC)	20.0	0	0	61	39	0
WB106 (pMCS104613C2)	0	0	0	0	0	0
WB106 (pMCS104613C2 + pACYC104MaoC)	10	0	12	38	49	0

As evident from Table 1 above, MCL-PHA was produced in the control group *E. coli* strain, and the test group *E. coli* strains WB101 (pMCS104613C2) and WB101 (pMCS104613C2 + p10499MaoC), but not

produced in the *E. coli* strains WB106 (pMCS104613C2) from which the *maoC* gene had been deleted. However, since MCL-PHA was produced in the recombinant *E. coli* WB106 (pMCS104613C2 + pACYC104MaoC) where the *maoC* gene was cloned into the *maoC* gene-deleted recombinant *E. coli*, it could be found that the m*aoC* gene played an important role in the production of MCL-PHA.

Langenbach et al. reported that MCL-PHA was produced in a *fadB* mutant *E. coli* strain transformed with recombinant vector pBHR71 containing a $phaC1_{Pa}$ gene, at a content of 21.1%(w/w) of cell dry weight (Langenbach et al., *FEMS Microbiol. Lett.*, 150:303-9, 1997). Tsuge et al. reported that MCL-PHA was produced in a recombinant *E. coli* strain expressing the PHA synthase gene originated from *Pseudomonas* sp. and the (R)-specific enoyl-CoA hydratases PhaJ1 and PhaJ2 originated from *Pseudomonas* sp., at contents of 14 and 29% (w/w) of cell dry weight, and the produced PHA mainly consisted of monomers with 6 carbon atoms (Tsuge et al., *FEMS Microbiol. Lett.*, 184:193-8, 2000).

In comparison with this prior art, the present invention allowed MCL-PGA to be produced at a higher content (44.6% (w/w)) than the prior art (14, 21.1 and 29%(w/w)). Furthermore, MCL-PHA produced according to the prior art consisted mainly of monomers with 6 carbon atoms, whereas MCL-PHA produced according to the method of the present invention consisted of monomers with 6-10 carbon atoms (3HHx, 3HO and 3HD), and particularly contained a large amount of monomers with 8-10 carbon atoms (3HO and 3HD). Thus, it could be found that MCL-PHA produced according to the method of the present invention would be used in a significantly wide range of application fields as compared to PHA produced by the prior method.

Example 3: Measurement of enoyl-CoA hydratase activity

A PCR fragment was produced in the same manner as Example 1-2 except that primer His-Tag: 5'-gctctagattaatggtgatgatggt gatgatcgacaaaatcaccg-3' (SEQ ID NO: 11) was used in substitute for the *maoC* primer 2. Furthermore, the PCR fragment was inserted into a pTac99A vector containing a *tac* promoter digested with *Sac*I/*Xba*I, thereby recombinant vector pTac99MaoCH was constructed (see, FIG. 4). The pTac99A vector was constructed by digesting a pKK223-3 vector (Pharmacia Biotech. Co., Uppsala, Sweden) with *Pvu*II/*Eco*RI to give the *tac* promoter, and cloning the *tac* promoter into pTrc99A (Pharmacia Biotech. Co., Uppsala, Sweden) digested with *Pvu*II/*Eco*RI. FIG. 4 is a gene map of the recombinant vector pTac99MaoCH containing the *maoC* gene. *E. coli* DH5α was transformed with the recombinant vector pTac99MaoCH to produce recombinant *E. coli* DH5α (pTac99MaoCH). The *E. coli* strain DH5α was cultured in LB medium for one day, and added with 1mM IPTG to induce protein expression. The colony was collected, homogenized and applied on a NTA column (Qiagen Ni-NTA kit, USA) to obtain a purified MaoC protein containing 6 histidines.

The purified MaoC protein and 0.25 mM crotonyl-CoA as a substrate were added to and mixed with reaction buffer (50mM Tris-HCl, pH 8.0), and enoyl-CoA hydratase activity was measured by following a reduction in absorbance at 263 nm due to the hydration of crotonyl-CoA. In this case, the ε_{263} of enoyl-CoA thioester as a product was calculated at 6700 $M^{-1}cm^{-1}$. As a result, it could be found that the MaoC protein showed the activity of enoyl-CoA hydratase of 47.6 U/mg toward crotonyl-CoA.

Industrial Applicability

As described and proved above, when the *maoC* gene whose function has not been yet fully established is used according to the present invention, high quality PHA with a higher number of carbon atoms than the prior PHA can be produced at a higher efficiency. Thus, the present invention will be widely applied in the production of high quality PHA.

Furthermore, the *E. coli* WB101 showed the highest PHA production, and MCL-PHA could be produced by amplification of the *maoC* gene and the PHA synthase gene in the recombinant *E. coli* W3110 (control group) having the normal pathway for fatty acid degradation, although the synthesis of PHA was possible by the use of the *maoC* gene alone. Considering that PHA cannot be generally produced from a fatty acid as a carbon source in fed-batch culture using *fadB* gene-deleted *E. coli*, the inventive method for producing MCL-PHA through amplification of the *maoC* gene in recombinant *E. coli* having the normal pathway for fatty acid degradation can be applied for the mass production of MCL-PHA by fed-batch culture.